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Concomitant regulation of Ca^{2+} mobilization and G_{i3} expression in human erythroleukemia cells

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Abstract

In human erythroleukemia (HEL) cells, stimulation of α_2-adrenoceptors by adrenaline or neuropeptide Y Y_1 receptors by neuropeptide Y, concomitantly inhibit cAMP accumulation and stimulate mobilization of Ca^{2+} from intracellular stores via pertussis toxin-sensitive G-proteins. Treatment of HEL cells in chemically-defined, serum-free medium with 1.25% dimethylsulfoxide (DMSO) for 4 days, increased α_2-adrenoceptor number by 120%, while the neuropeptide Y receptor number was not significantly changed. In DMSO-treated HEL cells, Ca^{2+} elevations by adrenaline or neuropeptide Y were significantly reduced by 28% and 57%, respectively, while basal Ca^{2+} and elevations by thrombin or thapsigargin were not significantly altered. Adrenaline and neuropeptide Y-induced inhibition of forskolin-stimulated cAMP accumulation was not significantly altered upon DMSO treatment. While immunodetectable α-subunits of G_{i2} were not significantly changed by DMSO treatment, those of G_{i3} were reduced by 27%. Inactivation of pertussis toxin substrates by pertussis toxin treatment and inhibition of adrenaline or neuropeptide Y stimulated Ca^{2+} elevations were linearly correlated. These data are compatible with the idea that, in HEL cells, α_2-adrenoceptors and neuropeptide Y receptors couple to inhibition of adenylyl cyclase via G_{i2} while they couple to Ca^{2+} elevations via G_{i3}. © 1998 Elsevier Science B.V.

Keywords: G-protein; Ca^{2+} mobilization; α_2-Adrenoceptor; Neuropeptide Y receptor

1. Introduction

Inhibition of adenylyl cyclase is a ubiquitous signal transduction pathway of receptors coupling to guanine nucleotide-binding proteins, (G-proteins) of the G_i type. However, not all physiological responses elicited by such receptors can be explained on the basis of adenylyl cyclase inhibition (Limbird, 1988). Additional signaling responses of G_i-coupled receptors, which are restricted to certain cell types, include modulation of K^+ and Ca^{2+} channel activity (Brown and Birnbaumer, 1988; Dolphin, 1991). G_i-coupled receptors can also affect free intracellular ion concentration by mobilizing Ca^{2+} from intracellular stores. This pathway has first been demonstrated for neuropeptide Y Y_1 receptors (Feth et al., 1992; Michel et al., 1992a; Motulsky and Michel, 1988) and α_{2A}-adrenoceptors (Michel et al., 1989) on human erythroleukemia (HEL) cells. Thereafter, it was also found for neuropeptide Y receptors on vascular smooth muscle (Mihara et al., 1989) or SK–N–MC neuroblastoma cells (Michel et al., 1992b), or with muscarinic M_2 acetylcholine receptors, heterologously expressed in 293 cells (Schmidt et al., 1995). These Ca^{2+} elevations occur via pertussis toxin-sensitive G-proteins and do not involve influx of extracellular Ca^{2+}. They occur independent of cAMP lowering, cyclooxygenase and lipoxygenase metabolites of arachidonic acid, and of Na^+/H^+-exchange. While some receptor systems, e.g., thrombin receptors in HEL cells, can cause Ca^{2+} elevations via pertussis toxin-sensitive G-proteins by activating a phospholipase C (Brass et al., 1991; Michel et al., 1989), the above agonist-stimulated Ca^{2+} elevations surprisingly appear to occur independently of inositol phosphates; thus, little if any concomitant inositol phosphate formation has been seen and phospholipase C inhibitors did not block them. Therefore, the exact pathways mediating Ca^{2+} elevations in response to neuropeptide Y receptor and α_2-receptor stimulation in HEL cells have remained elusive.

We have recently reported on mechanisms involved in the rapid desensitization of G_i-mediated Ca^{2+} mobilization in HEL cells (Michel, 1994). As part of these studies, we

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have observed that a four day treatment of HEL cells with 1.25% dimethylsulfoxide (DMSO) in chemically-defined, serum-free medium reduces Ca^{2+} mobilization via α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors. While DMSO is a rather non-specific agent, it has previously been shown that it induces the differentiation of HEL cells into a more platelet-like phenotype; this is accompanied by an enhanced agonist-induced desensitization of prostaglandin receptors (Ashby et al., 1991). In the present study, we have investigated in more detail which part(s) of the cellular signaling machinery are altered by DMSO treatment of HEL cells.

2. Material and methods

2.1. Cell culture

HEL cells were originally obtained from Dr. Papayannopoulou (Dept. of Medicine, University of Washington, Seattle, WA) and maintained in the chemically defined CG medium (Camon, Wiesbaden, Germany) in an atmosphere of 95% air/5% CO_2 at 37°C at a density of 400 000–600 000 cells/ml by daily dilution with fresh medium. During the last 4 days prior to the experiments, they were subcultured in the absence and presence of 1.25% DMSO. In some cases, the cells were treated with the indicated concentrations of pertussis toxin for the last 24 h prior to the experiment in the absence of DMSO.

2.2. Ca^{2+} measurements

The free intracellular Ca^{2+} concentration was determined with the fluorescent indicator dye Fura-2 in a Hitachi F2000 spectrofluorometer, as previously described (Feth et al., 1992). Briefly, experiments were performed in a buffer of the following composition (mM): Hepes 20, NaCl 120, KH_2PO_4 5, magnesium acetate 1, $CaCl_2$ 1 and 1 mg/ml glucose at pH 7.4 using approximately 600 000–800 000 cells/ml. After 1 h of loading with the dye, the cells were washed twice, resuspended in fresh buffer, and used for fluorescence measurements within the next hour. Excitation was alternating at 340 and 380 nm, with emission being read at 510 nm. Fluorescence data were converted into Ca^{2+} concentrations using software supplied by the manufacturer which uses the equation described by Grynkiewicz et al. (1985).

$$[Ca^{2+}] = K(R - R_{min})/(R_{max} - R)F_{min380}/F_{max380}$$

where K is the affinity constant of Fura-2 for Ca^{2+} (224 nM), R is the ratio of fluorescence determined at 340 nm excitation over that at 380 nm, R_{max} is the ratio determined when the dye is saturated with Ca^{2+} (determined after cell lysis with 0.02% digitonin), R_{min} is the ratio determined when free Ca^{2+} is absent (determined after chelation of all Ca^{2+} by addition of 10 mM ethylenediaminetetraacetic acid (EDTA) subsequent to cell lysis), and F_{max380} and F_{min380} are the fluorescence at 380 nm excitation following cell lysis and Ca^{2+} chelation, respectively. In these experiments, all drugs were added to the cell suspension in 100-fold concentrated stock solutions.

2.3. cAMP measurements

Cyclic AMP accumulation was determined during a 2 min incubation in the presence and absence of agonists, as previously described (Michel et al., 1989; Motulsky and Michel, 1988). The accumulated amount of cAMP was quantified by a commercially available protein binding assay (Amersham, Braunschweig, Germany).

2.4. Radioligand binding

Binding studies with HEL cell α_{2A}-adrenoceptors were performed in a membrane preparation, as described previously (Erdbrügger et al., 1995). Briefly, [^{3}H]RX 821002 (2-methoxy-idazoxan) binding was performed in a total volume of 250 μl of buffer (50 mM Tris, 0.5 mM EDTA, pH 7.5) for 60 min at 25°C and terminated by rapid vacuum filtration over GF/C filters. Non-specific binding was defined as binding in the presence of 10 μM phentolamine. Receptor number and affinity were determined by saturation binding experiments with 6 concentrations of the ligand. Data were analyzed by fitting rectangular hyperbolic function to the experimental data to obtain estimates of K_d and B_{max}.

Binding studies with HEL cell neuropeptide Y receptors were performed on intact cells, as described previously (Feth et al., 1992), except that the endogenous neuropeptide Y analog peptide YY was used as the radioligand since [^{125}I]peptide YY yielded less non-specific binding than [^{125}I]neuropeptide Y (data not shown). Briefly, [^{125}I]peptide YY binding was performed in a total volume of 500 μl of binding buffer of the following composition (mM): Hepes 10, NaCl 150, KCl 5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and 10 mg/ml bovine serum albumin. Additionally, the following protease inhibitors were added to the incubation mixture: 1 μg/ml aprotein, 40 μM amastatin, 10 μg/ml leupeptid, 10 μM pepstatin, 1 mM phenylmethanesulfonylfluoride, 50 μg/ml typsin inhibitor. Binding was carried out for 90 min at 25°C and terminated by centrifugation for 5 min at 3000 $\times g$. Non-specific binding was defined by binding in the presence of 100 nM neuropeptide Y. Receptor number and affinity were determined by homologous competition binding with unlabeled peptide YY and analyzed as described (DeBlasi et al., 1989).

2.5. Immunoblotting

The abundance of G-protein α-subunits was determined by quantitative Western blotting, as previously described (Michel-Reher et al., 1993). Briefly, membrane prepara-

tions (≈100 μg protein/sample) were diluted 4:1 with sample buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris–HCl, pH 8.0), boiled for 5 min, and separated on sodium dodecyl sulphate/polyacrylamide gels with 10% acrylamide in the running gel. The separated proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham, Braunschweig, Germany) with an electric field at a voltage of 55 V overnight. Following the transfer, the blots were washed for 90 min in TBS (20 mM Tris, 100 mM NaCl, pH 7.5) at room temperature, in the presence of 2% non-fat dry milk and twice for 10 min each in TTBS (TBS supplemented with 500 μl/l Tween-20), and then incubated overnight at 4° in 15 ml of TTBS containing 1% non-fat dry milk and a 1:500 dilution of the antisera. After removal of the antisera, the blots were washed twice for 10 min each with TTBS and then incubated for 1 h at room temperature in 100 ml of TTBS, which had been supplemented with 1% non-fat dry milk and 70 μl [^{125}I]protein A solution. Finally, the [^{125}I]protein A was washed out 4 times for 10 min each with TTBS and the blots were used for autoradiography at −80°C. Using the autoradiograms, the molecular weights of the specific bands were identified and corresponding sections were cut from the blots and counted in a γ-counter.

2.6. Pertussis toxin-catalyzed ADP-ribosylation

ADP ribosylation experiments were performed as previously described (Maisel et al., 1990). Briefly, membranes from cells which had been cultured in the absence and presence of pertussis toxin were incubated with 1 μM [^{32}P]nicotinamide adenine dinucleotide (NAD) and fresh pertussis toxin for 1 h at 30°C. The ribosylation reaction was stopped by addition of sampling buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris–HCl at pH 8.0) and subsequent boiling. The reaction products were separated by electrophoresis on sodium dodecyl sulphate gels containing 17% polyacrylamide. Specific phosphorylation bands were identified on autoradiograms of the dried gels, and ^{32}P incorporation was quantified by cutting appropriate parts from the gels and counting them in a scintillation counter. ^{32}P incorporation in lanes that had been loaded with membranes, previously incubated with ^{32}P, but without pertussis toxin, served as control and was subtracted from the data obtained in the other lanes in order to obtain specific ^{32}P incorporation.

2.7. Chemicals

[^{3}H]RX 821002 (specific activity 40–70 Ci/mmol) and [^{32}P] NAD (specific activity 30 Ci/mmol) were obtained from Amersham (Braunschweig, Germany) and [^{125}I]peptide YY (specific activity 2200 Ci/mmol) from Biotrend (Cologne, Germany). (−)-Adrenaline bitartrate, (±)-propranolol HCl and thrombin were from Sigma (Munich, Germany), human neuropeptide Y from Saxon (Hannover, Germany), thapsigargin from Calbiochem (La Jolla, CA, USA), pertussis toxin from List (Campbell, CA, USA), Fura-2-acetoxymethyl ester from Molecular Probes (Eugene, OR, USA), and G-protein antisera from New England Nuclear (Dreieich, Germany). Phentolamine mesylate was a kind gift of Novartis (Basel, Switzerland).

2.8. Data analysis

Data are given as mean ± S.E.M. of n experiments. Statistical significance of differences between means was assessed by paired two-tailed t-tests. Statistical calculations were performed using the Instat program (Graphpad Software, San Diego, CA, USA), and a $P < 0.05$ was considered significant.

3. Results

DMSO treatment increased the density of HEL cell α_{2A}-adrenoceptors by 120% ($n = 6$; $P = 0.014$; Fig. 1), while the affinity for the ligand was not significantly changed (479 ± 59 pM in control vs. 662 ± 117 pM in DMSO-treated cells; $P = 0.311$). The number of specific [^{125}I]peptide YY binding sites increased only slightly upon DMSO treatment and this difference did not reach statistical significance with the given number of experiments ($n = 5$; $P = 0.276$; Fig. 1); similarly the affinity for the agonist, peptide YY, was not significantly altered by DMSO treatment (1.78 ± 0.27 nM in control vs. 4.09 ± 1.15 nM in treated cells; $P = 0.084$).

DMSO treatment did not significantly alter basal Ca^{2+} concentrations (76 ± 2 nM in control vs. 81 ± 2 nM in treated cells; $n = 54$; $P = 0.101$). It also failed to significantly affect Ca^{2+} elevations caused by 0.1 U/ml throm-

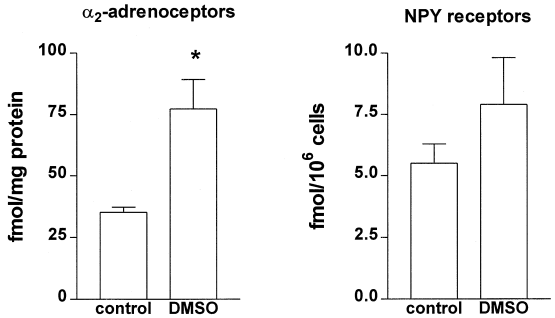

Fig. 1. Regulation of α_{2A}-adrenoceptor and neuropeptide Y Y_1 receptor expression by a 4 day treatment with 1.25% dimethylsulfoxide. Shown are densities of α_{2A}-adrenoceptors in HEL cell membranes as determined by [^{3}H]RX 821002 saturation binding (left panel) and neuropeptide Y (NPY) Y_1 receptors as determined by homologous competition for [^{125}I]peptide YY binding (right panel). Data are mean ± S.E.M. of 5–6 experiments in dimethylsulfoxide (DMSO)- and vehicle-treated cells (control). *: $P < 0.05$ vs. control in a paired two-tailed t-test.

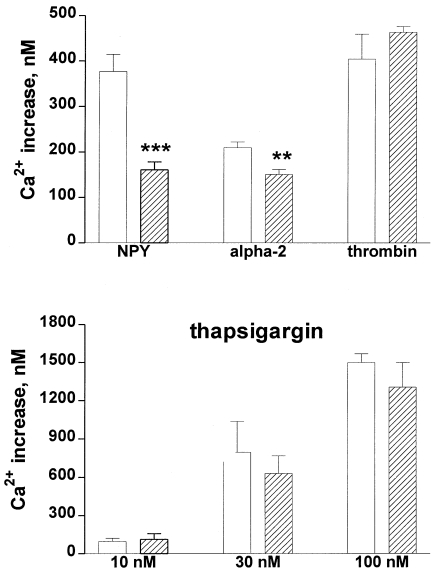

Fig. 2. Effects of a 4 day treatment with 1.25% dimethylsulfoxide on Ca^{2+} elevations in HEL cells. Shown are Ca^{2+} increases (expressed in nM) caused by agonist stimulation of neuropeptide Y (NPY) receptors by 100 nM neuropeptide Y, of α_{2A}-adrenoceptors (alpha-2) by 1 μM adrenaline, and of thrombin receptors by 0.1 U/ml thrombin (upper panel) and receptor-independently by 10, 30 and 100 nM thapsigargin (lower panel). Basal intracellular Ca^{2+} was similar in dimethylsulfoxide (DMSO)- and vehicle-treated cells (control). Data are mean ± S.E.M. of 6–15 experiments. ∗∗ and ∗∗∗: $P < 0.01$ and < 0.001, respectively, vs. control in a paired two-tailed *t*-test.

bin or by the Ca^{2+}-ATPase inhibitor, thapsigargin (10–100 nM; Fig. 2). In contrast, DMSO treatment significantly reduced Ca^{2+} mobilization by 1 μM adrenaline and 100 nM neuropeptide Y by 28% and 57%, respectively (Fig. 2).

The 10 μM forskolin-induced cAMP accumulation in DMSO-treated HEL cells (104 ± 11 pmol/2 min/10^6 cells) was significantly smaller than in control cells (178 ± 27 pmol/2 min/10^6 cells; $n = 5$; $P = 0.0241$). Inhibition of cAMP accumulation by 100 nM neuropeptide Y or 1

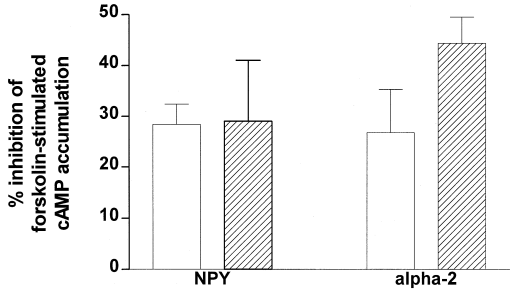

Fig. 3. Effects of a 4 day treatment with 1.25% dimethylsulfoxide on inhibition of forskolin-stimulated cAMP accumulation in HEL cells. Shown are effects of 100 nM neuropeptide Y (NPY) and 1 μM adrenaline (alpha-2) on cAMP accumulation stimulated by 10 μM forskolin during a 2 min incubation. Forskolin-stimulated cAMP accumulation was not significantly different between dimethylsulfoxide (DMSO)- and vehicle-treated cells (control). Data are mean ± S.E.M. of 5 experiments.

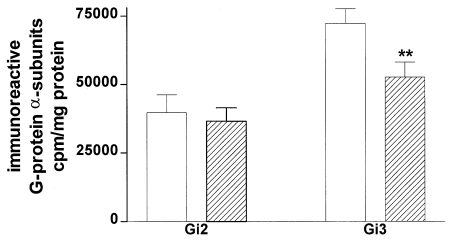

Fig. 4. Effects of a 4 day treatment with 1.25% dimethylsulfoxide on abundance of immunodetectable G-protein α-subunits in HEL cells. Shown are immunodetectable α-subunits of G_{i2} and G_{i3} in dimethylsulfoxide (DMSO)- and vehicle-treated cells (control). Data are mean ± S.E.M. of 7 experiments. ∗∗: $P < 0.01$ vs. control in a paired two-tailed *t*-test.

μM adrenaline (in the presence of 10 μM propranolol) was not significantly altered (Fig. 3).

In Western blots, the antisera AS/7 and EC/2, which specifically recognize $G_{i\alpha 1/2}$ and $G_{i\alpha 3}$, respectively, labeled bands with apparent molecular weights of 41 kDa (data not shown). The amount of immunodetectable $G_{i\alpha 2}$ was not significantly affected by DMSO treatment, while immunodetectable $G_{i\alpha 3}$ decreased in each of seven paired experiments with an overall reduction of approximately 27% ($P = 0.008$; Fig. 4).

A 24 h treatment with 0.03, 0.1 and 0.3 ng/ml pertussis toxin reduced the ability of freshly added pertussis toxin to cause ADP-ribosylation of its substrate in the membrane preparations by 60 ± 16%, 76 ± 4% and 89 ± 9%, respectively ($n = 8$). Pertussis toxin treatment also reduced α_{2A}-adrenoceptor and neuropeptide Y Y_1 receptor mediated Ca^{2+} elevations in a concentration-dependent manner and suppressed it almost completely at 0.3 ng/ml. A comparison of the potency of pertussis toxin for both effects demonstrated a linear correlation with a slope close to

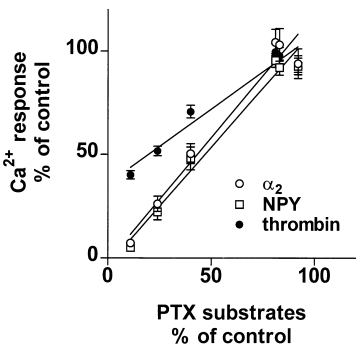

Fig. 5. Relationship between ADP-ribosylation of G_i proteins and inhibition of Ca^{2+} elevations in pertussis toxin-treated cells. HEL cells were treated with 0.003–0.3 ng/ml pertussis toxin (PTX) for 24 h. Thereafter, remaining pertussis toxin substrates were quantified in a membrane preparation of the treated cells using fresh PTX and [^{32}P]NAD. Other aliquots of the treated cells were used for measurements of Ca^{2+} elevations stimulated by 1 μM adrenaline (α_2), 100 nM neuropeptide Y (NPY) or 0.1 U/ml thrombin. Data are expressed as % of PTX substrates and Ca^{2+} elevations observed in parallel vehicle-treated cells and are mean ± S.E.M. of 6–8 experiments.

unity (Fig. 5). In contrast the Ca^{2+} elevations by thrombin were only partially inhibited even at the highest pertussis toxin concentrations (Fig. 5).

4. Discussion

We have previously demonstrated that treatment with 1.25% DMSO for 4 days, reduces α_{2A}-adrenoceptor and neuropeptide Y Y_1 receptor-mediated Ca^{2+} elevations in HEL cells (Michel, 1994). The possible underlying mechanisms of this reduction were investigated in more detail in the present study. In our initial experiments, we quantified the effect of DMSO treatment on receptor expression. For quantification of HEL cell α_{2A}-adrenoceptors, we used [^{3}H]RX 821002 as the radioligand because this ligand, in contrast to e.g., [^{3}H]rauwolscine, labels all HEL cell α_{2A}-adrenoceptors (Erdbrügger et al., 1995). Quantification of neuropeptide Y receptors in radioligand binding is hampered by the fact that only labeled agonists are available as high-affinity radioligands for this receptor. Since the use of labeled agonist as the radioligand in membrane preparations may detect only the agonist high-affinity sites of the receptor, we have performed our [^{125}I]peptide YY binding studies with intact HEL cells. Our data demonstrate that DMSO treatment increases HEL cell α_{2A}-adrenoceptor expression, while, that of neuropeptide Y Y_1 receptors is altered only slightly if at all.

The concentrations of adrenaline and neuropeptide Y used as agonists for the Ca^{2+} and cAMP experiments in the present study, represent maximally effective concentrations, as determined previously (Michel et al., 1989; Motulsky and Michel, 1988). In confirmation of our previous findings (Michel, 1994), DMSO treatment reduced Ca^{2+} mobilization by adrenaline and neuropeptide Y with reductions being greater for the latter than the former. This differential regulation of the Ca^{2+} response may be related to the increased α_{2A}-adrenoceptor expression following DMSO treatment. The reduced Ca^{2+} mobilization despite unchanged or even increased receptor expression was not a non-specific effect, since basal Ca^{2+} concentrations, elevations caused by the receptor agonist thrombin, which involves phospholipase C activation and influx of extracellular Ca^{2+} (Brass et al., 1991; Michel et al., 1989), or receptor-independently by the Ca^{2+}-ATPase inhibitor, thapsigargin, were not affected. Thus, the reduced Ca^{2+} mobilization following agonist stimulation of α_{2A}-adrenoceptors or neuropeptide Y Y_1 receptors is not explained by reduced expression of the respective receptors or by a general alteration of cellular Ca^{2+} handling.

Our next set of experiments addressed the question whether the DMSO-induced desensitization of Ca^{2+} signaling also affects inhibition of adenylyl cyclase by α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors. Our data demonstrate that this signal transduction pathway was not impaired by DMSO treatment. Taken together, these data indicate that the smaller Ca^{2+} elevation by α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors in DMSO-treated cells is not explained by reduced receptor numbers or a general impairment of their signal transducing capability.

Therefore, we investigated whether DMSO treatment affected expression of G-protein α-subunits which couple α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors to these effectors. We have previously shown that inhibition of adenylyl cyclase and Ca^{2+} mobilization by α_{2A}-adrenergic and neuropeptide Y Y_1 receptors occur via pertussis toxin-sensitive G-proteins (Michel et al., 1989; Motulsky and Michel, 1988). $G_{i\alpha 2}$ and $G_{i\alpha 3}$ are the only pertussis toxin substrates in HEL cells since $G_{i\alpha 1}$ and forms of G_o are absent (Michel et al., 1989; Williams et al., 1990). Our immunoblot data demonstrate that DMSO treatment does not affect immunodetectable quantities of $G_{i\alpha 2}$ but reduces those of $G_{i\alpha 3}$.

Thus, after careful exclusion of other possibilities, we propose that the reduced expression of $G_{i\alpha 3}$ may be the molecular basis of reduced Ca^{2+} mobilization by α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors following DMSO treatment. It may be argued that a 27% reduction in $G_{i\alpha 3}$ may not be sufficient to explain the reduced Ca^{2+} elevations in DMSO-treated cells if expression of the G-protein α-subunit considerably exceeds expression of the respective receptors (Alousi et al., 1991; Post et al., 1995). Therefore, we have compared the ability of pertussis toxin to inactivate HEL cell G_i-proteins and to inhibit α_{2A}-adrenoceptor and neuropeptide Y Y_1 receptor mediated Ca^{2+} elevations. Our data demonstrate that reduction of functional G_i α-subunits and inhibition of Ca^{2+} responses for α_2-adrenoceptors and neuropeptide Y Y_1 receptors occur with a 1:1 relationship. Since $G_{i\alpha 3}$ expression in HEL cells is less than $G_{i\alpha 2}$ expression (Williams et al., 1990), it may further be argued that our pertussis toxin data may not be representative for $G_{i\alpha 3}$. However, the acceptor sites of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ for pertussis toxin-induced ADP-ribosylation are almost identical. Accordingly, it has been found that both G-protein α-subunits have a very similar susceptibility for pertussis toxin-induced ADP-ribosylation (Graber et al., 1992; Linder et al., 1990). Moreover, peptides encompassing the last 10–20 amino acids of either G-protein have a sensitivity for pertussis toxin which is very similar to that of the intact protein, and peptides derived from $G_{i\alpha 2}$ and $G_{i\alpha 3}$ have a very similar affinity (Graf et al., 1992). Taken together, these data suggest that a 27% reduction in a G-protein α-subunit, as observed for $G_{i\alpha 3}$ upon DMSO treatment, can sufficiently explain a decrease in Ca^{2+} elevations by a similar magnitude. In light of the data scatter for measurements of immunodetectable $G_{i\alpha 3}$ and for neuropeptide Y-induced Ca^{2+} elevations, we propose that a reduced expression of the former is the most likely explanation of the latter.

Since inhibition of cAMP accumulation was not affected by DMSO treatment, the present data also indicate that $G_{i\alpha 3}$ may be less important for mediation of adenylyl

cyclase inhibition and that this may be brought about predominantly by $G_{i\alpha 2}$. A specific role for $G_{i\alpha 2}$ in coupling receptors to adenylyl cyclase inhibition has also been proposed for other model systems, e.g., for α_{2A}-adrenoceptors endogenously expressed in human platelets (Simonds et al., 1989) or in HT29 cells (Weiss and Littman, 1994) or those transfected into fibroblasts (McClue et al., 1992). Moreover, inhibition of cAMP accumulation via neuropeptide Y receptors has been blunted upon transfection with $G_{i\alpha 2}$ but not $G_{i\alpha 3}$ antisense constructs in a murine proximal renal tubule cell line (Voisin et al., 1996). Finally, antisense experiments in pituitary cells (Liu et al., 1994) and data in $G_{i\alpha 2}$ knock-out mice (Rudolph et al., 1996) have also indicated a dominant role for $G_{i\alpha 2}$ in adenylyl cyclase inhibition. Therefore, a review of published studies has suggested that $G_{i\alpha 2}$ may be the primary G-protein α-subunit to link α_2-adrenoceptors and possibly other receptors to adenylyl cyclase inhibition, although, other G-protein α-subunits may substitute under few selected conditions (Raymond, 1995). $G_{i\alpha 2}$ may also be responsible for α_{2A}-adrenoceptor coupling to voltage-dependent Ca^{2+} channels in rat portal vein despite the additional presence of $G_{o\alpha 2}$ and $G_{i\alpha 3}$ (Macrez-Lepretre et al., 1995).

Based on these data, we hypothesize that in HEL cells α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors couple in parallel to $G_{i\alpha 2}$ and $G_{i\alpha 3}$ with the former preferentially coupling to adenylyl cyclase inhibition and the latter preferentially to Ca^{2+} mobilization. Partial agonists at HEL cell α_{2A}-adrenoceptors or neuropeptide Y Y_1 receptors have a smaller relative efficacy for Ca^{2+} mobilization than for inhibition of cAMP accumulation (Michel et al., 1989, 1990). Since little amplification is believed to occur between receptors, G_i-proteins and adenylyl cyclase inhibition and since $G_{i\alpha 3}$ expression in HEL cells is less than $G_{i\alpha 2}$ expression (Williams et al., 1990), we speculate that the different efficiency of signal transduction between receptors and adenylyl cyclase inhibition on the one and Ca^{2+} mobilization on the other hand might result from a differentially effective coupling of HEL cell α_{2A}-adrenoceptors and neuropeptide Y Y_1 receptors to $G_{i\alpha 2}$ and $G_{i\alpha 3}$

The idea of parallel receptor coupling to various G-proteins with a subsequent relative specificity of coupling to an individual signaling response is also supported by other studies. Thus, in GH4C1 pituitary cells D_2 dopamine receptors couple to Ca^{2+} channel inhibition via G_o and to adenylyl cyclase inhibition via G_{i2} (Liu et al., 1994). Purinergic receptor stimulation of CHO cells elevates intracellular Ca^{2+} and activates cytosolic phospholipase A_2 and mitogen-activated protein kinase in a pertussis toxin-sensitive manner; transfection with a dominant negative mutant of G_{i2} inhibits purinergic stimulation of phospholipase A_2 but not Ca^{2+}, elevations or activation of mitogen-activated protein kinase (Murray-Whelan et al., 1995). Moreover, cotransfection of *Xenopus laevis* oocytes with neuropeptide Y Y_1 receptors and pertussis toxin-sensitive G-protein α-subunits activates an inward rectifying K^+ channel when $G_{i\alpha 1}$ but not when $G_{i\alpha 2}$, $G_{i\alpha 3}$ or $G_{o\alpha}$ are cotransfected (Brown et al., 1995). Thus, a given receptor can activate multiple G-proteins which then specifically regulate distinct effector mechanisms.

While a reduction of $G_{i\alpha 3}$ is the simplest explanation of reduced Ca^{2+} responses upon DMSO treatment according the present data, it has not escaped our attention that such coregulation studies cannot provide definitive evidence for specific coupling events between receptors, G-proteins and effector mechanisms. While that was not the aim of the present study, it might be suggested that a more definitive answer is obtained by either eliminating or overexpressing one of the G-protein α-subunits in question. However, we feel that this approach is also problematic since the selectivity of receptor/G-protein/effector interactions is only relative, and therefore marked alterations of the ratios of these components might yield promiscuous coupling, which does not occur at physiological expression levels. For example, endogenous G_{i2} α-subunits appear to mediate adenylyl cyclase activity under normal circumstances (Liu et al., 1994; Simonds et al., 1989; Weiss and Littman, 1994), while transfected G_{i3} α-subunits can do so when all endogenous pertussis toxin substrates have been inactivated (Hunt et al., 1994). Moreover, a complete knock-out of one pertussis toxin substrate, e.g., $G_{i\alpha 2}$, may lead to compensatory overexpression of another pertussis toxin substrate, e.g., $G_{i\alpha 3}$, as has been observed in $G_{i\alpha 2}$ knock-out mice (Rudolph et al., 1996). Therefore, we suggest that overexpression and/or knockout experiments could complement but do not substitute for studies such as the present, since marked alterations of stoichiometric ratio of receptors, G-proteins and effector molecules may result in promiscuous coupling.

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